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Preliminary Evaluation of a Multiplex Reverse Transcription-PCR Assay Combined with a New DNA Chip Hybridization Assay for Detecting Respiratory Syncytial Virus▽

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DNA chips represent a major advance in microbiology laboratories, enabling the detection of a wide range of possible pathogens using a single test. This study compared a multiplex reverse transcription-PCR combined with DNA chip hybridization (ProDect BCS RV chip; bcs Biotech) with the indirect immunofluorescence test commonly used to detect respiratory viruses. A total of 39 respiratory viruses (38 respiratory syncytial viruses [RSVs] and 1 influenza A virus) were detected in samples from 96 patients using the immunofluorescence test, while 36 viruses (34 RSV, 1 influenza A virus, and 1 influenza B virus) were detected by the DNA chip technique. Results showed a good level of agreement between the two tests for RSV detection; the incidence of other viruses was low, since samples were taken from patients with suspected bronchiolitis. DNA chips displayed high sensitivity (94.6%) and specificity (100%).

Respiratory virus infections in infants and preschool children may be caused by a number of microorganisms; several viruses may be involved (all of them RNA viruses except for adenovirus), chief among which are respiratory syncytial virus (RSV), adenovirus, influenza A and B viruses, parainfluenza virus types 1, 2, and 3, coronavirus, and the recently identified metapneumovirus. These viruses can cause a wide range of pathologies, the mildest of which, rhinitis and pharyngitis, are relatively minor, except for their ease of spread through fomites, hands, or Pflüger droplets. However, in neonates and children under 2, these viruses, particularly RSV, can cause bronchiolitis, which is associated with higher disease and mortality rates, when the defensive inflammatory response to infection blocks the tiny airways of infant patients, although recent studies attribute serious cases to inadequate (rather than excessive) adaptive immune responses, robust viral replication, and apoptotic crisis (14), leading to hospitalization, in some cases the need for assisted ventilation, and patient isolation to prevent the disease from spreading to other patients in pediatric intensive care units. Pneumonia is also relatively common in infants, as well as in immunocompromised subjects and in elderly patients with comorbidities.

Detection of these viruses has traditionally involved isolation in culture medium, a process associated with considerable diagnostic delay (up to 7 days); this is of particular concern given the acute nature of the disease. However, immunofluorescence (IF)-based shell vial centrifuge techniques reportedly improve the results obtained at 24 h (4).

The development of IF testing (specially when directly applied to clinical samples [10]) marked a breakthrough in virus identification, since rapid diagnosis helped to decrease mortality, reduce the length of hospitalization, and reduce hospital costs, as well as avoiding inappropriate antibiotic use (1, 15). The main drawback is the subjectivity of the technique, which is also dependent on observer experience.

Immunochromatographic assay has also been used to identify respiratory viruses and is reported to provide an emergency diagnosis in less than 15 min, with a high degree of specificity; unfortunately, its moderate sensitivity (<80%) requires the joint use of other techniques (6).

Preliminary research into the molecular diagnosis of respiratory viruses using PCR suggest that this technique is more sensitive than either culturing or fluorescent-antibody testing (7, 12, 13). Molecular diagnosis by DNA chip marks a technological advance in the identification of respiratory viruses.

The aim of this study was to evaluate a new DNA chip for the identification of respiratory viruses (ProDect BCS RV chip; bcs Biotech, Cagliari, Italy), which contains probes for RSV, adenovirus, parainfluenza virus types 1, 2, and 3, and influenza A and B viruses. Parallel testing was performed using indirect IF for the same viruses (we applied the commercial protocol supplied for direct IF examination, thus using the antibodies directly on samples taken from the patient without overnight culture); immunochromatography was used to check any discordant results in RSV detection by DNA chip or IF.

Nasopharyngeal lavage samples were obtained from 96 pediatric patients aged 2 months to 5 years admitted to the Hospital Universitario Reina Sofía (Córdoba, Spain) with suspected bronchiolitis between November 2005 and January 2006.

All specimens were subjected to indirect IF testing using a Bartels viral respiratory (Trinity Biotech, Bray, Ireland), which was performed and interpreted following the manufacturer's instructions for RSV, influenza A and B viruses, parainfluenza virus types 1, 2, and 3, and adenovirus (5), DNA chip testing for these viruses, and an immunochromatographic assay for RSV (Binax NOW, Scarborough, Maine); this test was used first, as it provides the clinician with a rapid response in hospital emergencies (9).

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Samples positive by at least two of the techniques were considered true positives, and samples negative by at least two of the techniques were considered true negatives.

Multiplex RT-PCR combined with DNA chip hybridization. All steps except extraction (not included in the ProDect BCS RV chip kit; bcs Biotech) were carried out using materials included in the ProDect BCS RV chip kit. The kit comprises two parts: part A consists of the reagents required for PCR (DNA polymerase, $MgCl_2$, deoxynucleoside triphosphates, PCR buffer, H_2O diethyl pyrocarbonate, and two RT-PCR mixes, the first containing biotinylated oligonucleotides and the second biotinylated oligonucleotides and plasmid DNA); part B comprises hybridization, washing, and detection buffers, blocking reagents, nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP), streptavidin alkaline phosphatase, and 96 chips grouped in threes.

The target regions used by the kit for parainfluenza virus types 1, 2, and 3 are the corresponding HN genes; NS genes are targeted for influenza A and B viruses, the H gene for adenovirus, and the NS2 gene for RSV.

The multiplex PCR on the DNA chip was performed as follows. (i) Genetic material extraction (NucliSens lysis buffer and automated isolation reagents; Biomerieux, Marcy l'Etoile, France). To extract RNA or DNA (for adenovirus), 200 μl of sample (nasopharyngeal lavage) was mixed with lysis buffer (900 μl) and incubated for 10 min at room temperature. For genome recovery, silica beads (50 μl) were added, with vortexing every 2 min for 10 min to facilitate binding. Next, tubes were centrifuged at 10,000 × *g* for 3 min, and the supernatant was discarded. Silica pellets were washed twice with guanidine thiocyanate; after each wash, the suspension was centrifuged for 1 min at 10,000 × *g*, and the supernatant was discarded. Pellets were then washed twice with 70% ethanol, centrifuged for 1 min at 13,000 × *g* after each wash, and finally washed in acetone and centrifuged for 1 min at 10,000 × *g*. The last supernatant was dried on a heat block at 56°C for 10 min, with caps open. Finally, 50 μl of elution buffer was added, and after vortexing, the mixture was incubated for 10 min on a heat block at 56°C, with vortexing every 2 min.

(ii) RT. For each sample, RT-PCR was performed using two Eppendorf microcentrifuge tubes with two different RT mixes, since the high number of different primers could lead to nonspecific reactions. Sample aliquots (4 μl) were placed in each tube, one of which contained 2 μl mix 1 and the other 2 μl mix 2. Beads were dissolved in 20 μl DEPC-treated water, and nucleotides (4 μl) were then added to each microcentrifuge tube. The reaction mixture was incubated in a thermal cycler at 42° for 45 min to obtain cDNA.

(iii) Amplification. Amplification was performed in the same microcentrifuge tubes, using 40 μl of a DNA polymerase solution. PCR comprised 35 cycles of 95°C for 5 min, 95°C for 20 min, 50°C for 20 min, and 72°C for 40 min, and then 72°C for 10 min.

(iv) Hybridization. Amplified material (20 μl) was placed in the DNA chip with 480 μl hybridization buffer and incubated at 45°C for 1 h to allow amplicons to bind to the appropriate probe. Three washes were performed in buffer to eliminate nonspecific hybridization; the chip was emptied after each wash.

TABLE 1. Comparison of DNA chip and Immunofluorescence testing

IF result	No. of samples positive by DNA chip				
	RSV[a]	Influenza A virus	Influenza B virus	Negative	Total
RSV[a]	32	0	0	6	38
Influenza A virus	0	1	0	0	1
Influenza B virus	0	0	0	0	0
Negative	2	0	1	55	58
Total	34	1	1	61	97[b]

[a] The kappa coefficient was 0.82 ($P < 0.001$).
[b] One sample was positive for influenza B virus and RSV by the DNA chip test.

(v) Detection reaction. The amplicon-probe complex was visualized by colorimetric reaction using a streptavidin-alkaline phosphatase (Strep-AP) system: 0.5 μl Strep-AP was added to 500 μl blocking reagent, and the mixture was allowed to sit for 30 min at room temperature. The chip was emptied and washed three times in 500 μl buffer, with emptying after each wash. Next, 49 μl detection buffer and 10 μl NBT-BCIP were added, and mixtures were allowed to sit for 10 min at room temperature in the dark. After a final wash in distilled water, results were read automatically on a chip scanner equipped with commercial software which interpreted all spots appearing on the array, identifying controls and probes revealed for the various viruses.

The DNA chip contained four hybridization and detection controls, located at the four corners of the chip, which ensure binding of amplified material to probes and the correct functioning of the Strep-AP-based colorimetric reaction, and one centrally located internal amplification control for validation purposes, together with the respiratory virus probes. All controls were revealed as spots appearing in a given position.

McNemar's χ^2 test was used for statistical analysis. The clinical agreement index (2) was also applied, and confidence intervals were set for a P value of <0.05. The main validity criteria (sensitivity, specificity, positive predictive value [PPV], and negative predictive value [NPV]) were measured using the EpiInfo 6,04d program (Centers for Disease Control and Prevention).

Results are shown in Table 1. A total of 36 respiratory viruses were detected using the DNA chip: 34 RSVs, one influenza A virus and one joint influenza B virus–RSV infection. IF testing detected 39 respiratory viruses: 38 RSVs and 1 influenza A virus. Thirty-two RSV samples were positive by both techniques, as was the sample with the only influenza A virus recovered. A total of 55 samples were negative for both tests. Parainfluenza virus types 1, 2, and 3 were not detected.

The DNA array recovered two RSVs classed by IF testing as negative but by immunochromatography as positive. The joint influenza B virus–RSV infection was detected only by DNA chip, being classified as RSV alone by IF. This case was excluded from statistical analysis, since no other means of detection was available for influenza B virus.

In six of the nasopharyngeal lavage specimens identified as positive by IF, the DNA chip detected no genetic material at all. In four of these, immunochromatography also yielded negative results (the IF results thus being deemed false positives); the other two cases were deemed DNA chip false negatives.

TABLE 2. Validity parameters for the techniques tested for RSV

Parameter	DNA chip		IF	
	Value (%)	95% confidence interval	Value (%)	95% confidence interval
Sensitivity	94.6	80.5, 90.1	94.6	80.5, 99.1
Specificity	100	92.4, 100.0	93.2	82.7, 97.8
PPV	100	87.7, 100.0	89.7	74.8, 96.7
NPV	96.7	87.6, 99.4	96.5	86.8, 99.4

The most notable validity parameters (Table 2) were the high specificity and PPV of the DNA chip and the high sensitivity and NPV of the IF tests. The kappa coefficient was 0.82 ($P < 0.001$), showing strong agreement between the two techniques.

These statistical tests were applied only to RSV, given the low incidence of other viruses in this study.

The IF and DNA chip techniques both proved absolutely valid for the detection of this set of RSVs, displaying very similar values for sensitivity and specificity; the results showed good agreement. The DNA chip performed well for other viruses, but given the low incidence of non-RSV viruses due to the type of samples received, only RSV could be analyzed.

Papers delivered at recent congresses (8, 11) also reported good sensitivity (88.2%) and specificity (98.5%) for RSV and similar values for other respiratory viruses. Another recent article (3), however, compared the chip assay with the combination of shell vial cell culture and IF antibody test for the detection of respiratory viruses. Those authors reported 25% as the global sensitivity for the DNA chip, a much lower rate than ours (94.6% for RSV) and than the ones provided by the manufacturer (72% to 93%, depending on the virus). They described the detection of nine RSVs with IF and only five with the ProDect BCS RV chip, with a 55.6% sensitivity, higher than that obtained for other respiratory viruses but still lower than ours and the manufacturer's. In addition, their extraction method is not the one we used.

The DNA chip technique avoids the subjectivity inherent to IF and is not influenced by observer experience, since results are read automatically (BCS AiM reader); however, it is more laborious and not wholly error free, since it is a highly manual process. It also takes longer than IF (a total time of about 6 h, compared to around 1.5 h). Most microbiologists would agree that the future of molecular microbiology lies in the DNA chip. In the early days of PCR, techniques were also largely manual and laborious; in the same way, future work in microarray technology should be aimed at automating the technique, in order to reduce the workload and increase throughput.

Further research is required, with larger sample sizes, to confirm the validity of this new technique.

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